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(71) *Sökande* *Ulf Landegren Inst f medicinsk genetik, Uppsala SE*
Applicant (s)

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Evy Morin
Evy Morin

Avgift
Fee

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

NOVEL USE OF PADLOCK PROBES

The present invention relates to compositions for targeting nucleic acid sequences, more specifically double stranded nucleic acid sequences. The compositions comprise oligonucleotides in the form of padlock probes. Furthermore, the invention relates to use of said compositions in a method for directly inhibiting gene function by binding to double stranded nucleic acids.

Oligonucleotides as potential therapeutics has developed by the ability to synthesize oligonucleotides, chemically modified oligonucleotide analogs and conjugated oligonucleotides, of suitable quantity and purity, as a result of the now ready availability of oligonucleotides through automated synthesis using, for example, the phosphoramidite method.

A first approach to therapeutic use of oligonucleotides is to use them as inhibitors of translation, with the complementary or 'antisense' base sequence targeted to a specific 'sense' sequence in the mRNA. In this way, expression of a specific protein can be regulated or inhibited.

Mechanisms of antisense inhibition include interference with ribosome binding and processing of mRNA conformation or mRNA splicing, and RNAase-H activation of mRNA digestion. The preferred target for antisense inhibition is the 5'-initiation codon.

A second approach to therapeutic use of oligonuclectides is to target DNA therewith and thereby directly inhibit gene function by inhibiting transcription to mRNA. In contrast to mRNA which, although extensively folded, is readily accessible, the DNA duplex is very stable which complicates inhibition thereof.

One way of solving the problem with inaccessibility of double stranded DNA is to take advantage of the fact that a third strand can be accomodated in the major groove of the B-form DNA duplex

to form a triplex structure.

Duplex recognition by an oligonucleotide involves the formation of two hydrogen bonds with the purines of Watson-Crick base pairs within the major groove of the double helix. Thymine, cytosine, and guanine can adopt two different orientations called 'Hoogsteen' and 'reverse Hoogsteen' by analogy with the hydrogen-bonding scheme discovered by Hoogsteen in co-crystals of A and T derivatives. In contrast, adenine and inosine can form two hydrogen bonds with and A.T base pair in a single orientation. It should be noted that in order to form two hydrogen bonds with G, cytosine must be protonated. Therefore, triplets involving C+ x G.C are more stable at acidic pH. Methylation at C-5 of cytosine also contributes to stabilisation of the triple helix.

Several mechanisms exist by which triple helix formation can alter gene transcription:

1. Triple helix formation within the promoter region can change DNA conformation and therefore alter the rate and effiency of RNA polymerase initiation. This can lead to either activation or inhibition of transcription.
2. Oligonucleotide binding to a DNA sequence overlapping a transcription factor binding site may inhibit its transactivating capacity.
3. Triplex formation within or adjacent to the region where RNA polymerase binds may inhibit transcription initiation even if RNA polymerase and transcription factors are still bound to the promoter.
4. Oligonucleotide binding downstream of the RNA polymerase recognition site might inhibit progression of the transcription machinery along the DNA and therefore block RNA elongation.

Targeting by triple helix formation is limited to only a particular subset of DNA sequences, such as those associated with homopurine-homopyrimidine tracts.

An alternative way of directly inhibiting DNA is described in

Nucleic Acids Research, 1993, Vol 21, No 2, p 197-200 to Nielsen et al. The authors describe that PNA (peptide nucleic acids chimera), i.e., DNA analogues in which the deoxyribose phosphat backbone has been exchanged for a peptide backbone consisting of (2-amoniethyl)glycine units have retained the hybridization properties of DNA. There is shown that PNA binds more strongly to complementary oligonucleotides than DNA itself. Moreoever, PNA can bind sequence specifically to double stranded DNA. This binding takes place by strand displacement rather than by triple helix formation. In brief, a rather unstable strand displacement complex is first formed with only one PNA molecule bound to the target by Watson-Crick hydrogen bonding, and this is subsequently trapped by binding of a second PNA molecule via Hoogsteen hydrogen bonding.

However, because of their relatively strong binding the sequence specificity rapidly deminishes with the length of the PNA probes.

Squire et al in Angew. Chem. Int. Ed. Engl. 1992, 31, No. 12, describes circular hybrid molecules which contain two oligonucleotide domains bridged by two oligoethyleneglycol chains. These molecules bind with high affinity to complementary strands of RNA and DNA and display resistance to degradation by nucleases. However, these probes only bind to single stranded molecules.

Branch capture reactions (BCRs) target duplex restriction fragments terminating in overhanging bases with short homologous single stranded DNA oligonucleotides that can pair with the unpaired overhanging bases and some flanking sequence so that complete base pairing displaces the end of one resident strand by branch migration. The limitation of BCRs is that they are limited to targeting only known terminal sequences and are, thus, not very suitable as therapeutic agents.

In Nature Genetics, vol. 3, april 1993, there is described another probe-targeting method which uses Rec A protein-coated

short single stranded DNA probes to form four stranded hybrids between probes and duplex DNA targets. With this m thod internally localized sites can be targeted and the four stranded hybrids are stable.

All the above nucleic acid targeting methods suffer from drawbacks the most important one being the insufficient sequence specificity of the probes. This is an especially essential consideration in respect of the potential use of the probes as therapeutics.

The present invention is derived from the copending international application no. PCT/SE95/00163 entitled: Method, reagent and kit for detection of specific nucleotide sequences. This application is referred to and heirein incorporated by reference. In this application so called padlock probes are described.

In summary, said application describes a probe designed to be circularized in the presence of a target sequence, wherein said probe is caused to close around the target nucleic acid, for example DNA or RNA, such that the cyclic probe will interlock with and thereby be efficently linked to the target nucleic acid in a manner similar to "padlocks". The circularization of the probe ends is achieved with, for example, ligase. Such covalent catenation of probe molecules to target sequences result in the formation of an extremely stable hybrid.

It has now been surprisingly found that these padlock probes are able to affect gene function directly by binding to double stranded nucleic acids, without a prior denaturation step, and thereby affect the replication and transcription of the bound molecule.

The present invention provides nucleic acid targeting compositions having the desired properties described above. The compositions comprise an effective amount of a padlock probe. The padlock probe has two free nucleic acid end parts which are at

least partially complementary to and capabl of hybridizing with two at least substantially neighbouring respective regions of a target double stranded nucleic acid sequence. The padlock probe can be circularized and catenate with the target sequence in th presence of a linking agent. The compositions comprising the padlock probes have inhibiting activity on double stranded nuleic acids and are formulated with a carrier suitable for their intended use.

Furthermore, the present invention provides a method of inhibiting double stranded nucleic acids in which the abov described compositions are administred.

Padlock probe targeting to double stranded DNA involves a linking agent which can be chemical or biological. It is, for example, a ligase-assisted reaction. The principle employed in such a reaction is that two-probe segments, complementary to target sequences situated in juxtaposition, are joined to a contiguous probe sequence with the aid of a DNA ligase. Examples of ligases are T4 DNA ligase, T7 DNA ligase, E.coli DNA ligase, and Thermus thermophilus DNA ligase. Besides ligases, proteins like RecA or single strand-binding protein allow circularized probes to be complemented with, and catenated to, base paired DNA.

The compositions according to the invention may or may not contain a linking agent depending on the use of the compositions. In vivo, RecA and DNA ligase are already present, and thus the addition of a linking agent is not necessary for therapeutic applications.

According to a further embodiment of the present invention, padlock probes are used as in vitro reagents. For this application, the effect of the padlock probes is further improved by partially building the probes of altered nucleic acids, for example PNA, having stronger base pairing.

Padlock probes bind selectively and stably to double stranded DNA

and enable sequenc specific modification of DNA. In f ct, it is contemplated that padlock probes even will be able to inhibit genomic point mutations since the ligation is dependent upon the exac' target sequence. The increased specificity is achieved by the fact that two shorter probe segments have to cooperate for binding to occur. A further advantage is that padlock probes are not sensitive to exonucleases due to their circular shape when they are ligated. On the other hand, excess of padlock probes is rapidly degraded by exonucleases which is a benefit in, for example, drug formulation.

The invention will now be illustrated further, by way of example only, by the following non-limiting specific Example.

EXAMPLE

A padlock probe oligonucleotide having the following sequence: 5' P-TGG TGT TTC CTA TGA-((HEG$_2$)C-B)$_4$(HEG)$_2$-AAG AAA TAT CAT CTT-3', wherein P is a phosphate residue, HEG is hexaethylene glycol and C-B is a biotinylated C residue, was synthesized using a commercial DNA synthesizer. The two ends of the oligonucleotide were capable of base-pairing adjacent to each other with exon 9 of the CFTR gene contained in the double stranded plasmid pUC 19.

The probe was labelled by exchanging the present 5' phosphate residue with ^{32}P using polynucleotide kinase and was allowed to hybridize with the target sequence. In a volume of 20 µl 2 pmole probe were mixed with 0.2 pmole of plasmid in the presence of 24 pmole RecA protein in a solution of 10 mM Tris, pH 7.5, 10 mM Mg(Ac)$_2$, 50 mM KAc, 2 mM ATP with 5 units T4 DNA ligase and was incubated for 30 minutes at 37°C.

After incubation, washing was performed under non-hybridizing conditions. Thereafter, the reaction products were separated on a denaturing 6% polyacrylamide gel and the radioactive label was quantified with a Phosphorimager. The results clearly showed binding of the above padlock probe to the double stranded plasmid.

CLAIMS

1. A nucleic acid targeting composition, characterized in that it comprises an effective amount of a padlock probe having two free nucleic acid end parts which are at least partially complementary to and capable of hybridizing with two at least substantially neighbouring respective regions of a target doubl stranded nucleic acid sequence so that it can be circularized and catenate with the target sequence in the presence of a linking agent, wherein said composition has inhibiting activity on double stranded nucleic acids.

2. A nucleic acid targeting composition according to claim 1 in admixture with a suitable carrier.

3. A nucleic acid targeting composition according to claim 1 in admixture with a pharmaceutically acceptable carrier.

4. A composition according to anyone of the claims 1-3 for use in a method of directly inhibiting gene function by binding to a double stranded nucleic acid.

5. A method of inhibiting a double stranded nucleic acid, characterized by administration of a composition according to anyone of the claims 1-3.

ABSTRACT

The present invention relates to novel use of padlock probes in compositions for targeting nucleic acid sequences, more specifically double stranded nucleic acid sequences. Furthermore, the invention relates to use of said compositions in a method for directly inhibiting gene function by binding to double stranded nucleic acids.